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Ankyrin repeat and BTB/POZ domain containing protein-2 inhibits the aggregation of alpha-synuclein: Implications for Parkinson's disease

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ABSTRACT

Aggregation of α-synuclein is a pathological hallmark of sporadic or familial PD. However, the detailed molecular mechanism responsible for the aggregation of α-synuclein has not been properly explored. In the present study, we have identified a novel role of an anti-tumorigenic BTB/POZ domain containing protein-2 (BPOZ-2) in the regulation of α-synuclein accumulation in dopaminergic (DA) neurons. MPP^+, an etiological factor for PD, significantly downregulated the expression of BPOZ-2 ahead of α-synuclein upregulation. Moreover, siRNA knockdown of BPOZ-2 alone stimulated the aggregation of α-synuclein protein; the effect was further induced in presence of MPP^+ in mouse primary DA neurons. Finally, the absence of BPOZ-2 in α-synuclein expressing neuronal populations of MPTP-intoxicated mouse and primate nigra indicates that the suppression of BPOZ-2 could be involved in the accumulation of α-synuclein protein.

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1. Introduction

BTB/POZ domain containing protein is a growth suppressor protein [1] that mainly participates in protein–protein interaction with E3 ubiquitin ligase [2]. Its role as a scaffold protein mainly stems from the presence of ankyrin repeats (ARs) and two BTB/POZ domains that render BPOZ to function as an interface between two proteins [3]. BPOZ has three spliced variants named as BPOZ-1, 2, and 3 [4]. BPOZ-2 has two ARs, BPOZ-3 has one and BPOZ-1 has no AR. Evidently, BPOZ has been reported to interact with E3 ubiquitin ligase via its BTB domain and its interaction with one such E3 ubiquitin ligase Cul-3 [2] has been shown to arrest the growth of tumors. As a mechanism, the overexpression of BPOZ has been reported to arrest the transition of cell cycle from G1 to S phase [5]. Although, its role in the tumor biology is currently being studied, nothing is known regarding its involvement in the pathogenesis of neurodegenerative disorders. Among all three BPOZ proteins, BPOZ-1 and 2 has been shown to be present ubiquitously in the different parts of the brain.

Aggregation of α-synuclein is not only the hallmark of sporadic PD [6], but a reliable diagnostic marker of classical PD [7] as well. The α-synuclein containing Lewy bodies have been shown to be involved in the loss of DA neurons [8,9] in all form of PD. Although, the mechanism of α-synuclein aggregation is not clearly understood, the dysfunction of parkin, an E3 ubiquitin ligase, could be associated in the inhibition of the proteasomal degradation of α-synuclein protein leading to the formation of α-synuclein aggregates [10]. Recent studies indicate that the nitrosylation of parkin inhibits its catalytic activity [11], albeit its direct effect in the accumulation of α-synuclein is yet to be explored. Since, BPOZ controls the activity of E3 ubiquitin ligase protein, our present study explores if BPOZ has any role in the proteasomal degradation of α-synuclein aggregates in the nigra of MPTP-intoxicated mouse brain of PD. Our results clearly showed that the expression of BPOZ protein and mRNA was strongly downregulated in the MPP^+-treated DA neuronal culture as well as MPTP-treated mouse nigra. Our results further demonstrated that the selective knockdown of *BPOZ-2*gene sufficiently stimulated the aggregation of α-synuclein protein without altering the mRNA expression of *snca* gene indicating its direct and posttranscriptional role for the possible turnover of α synuclein aggregates.

2. Result

2.1. BPOZ-2 could be involved in the inhibition of α-synuclein aggregation in mouse primary DA neurons

MPP^+ is the etiological toxin of Parkinson's disease that has been reported to induce the expression of α-synuclein [12,13]

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protein. The exaggerated expression of α-synuclein protein forms cytoplasmic proteinaceous inclusions with other cytoskeletal proteins, which eventually paralyze the cellular metabolic process and finally cause the death of DA neurons [14]. Since, BPOZ-2 plays an important role in the clearance of protein in other cell types [4,5], we first wanted to study if BPOZ-2 was at all expressed in DA neurons. Interestingly, we have observed that DA neurons strongly express BPOZ-2 mRNA (Fig. 1A and B) and protein (Fig. 1C and D). Moreover, the expression of that protein is significantly disturbed in the presence of Parkinsonian toxin MPP^+ (Fig. 1A–F) as we found that MPP^+ inhibited the mRNA expression of BPOZ-2 with increasing doses (Fig. 1A and B) and time (Fig. 1E and F) suggesting the possible role of BPOZ-2 in the protection of DA neurons. However, the expression of BPOZ-1, another bpoz family protein, was not altered with the increasing concentrations of MPP^+ (Fig. 1A and B) suggesting the specific role of BPOZ-2 in the protection of DA neurons. The strongest effect on BPOZ-2 mRNA expression was observed at 45 min of stimulation

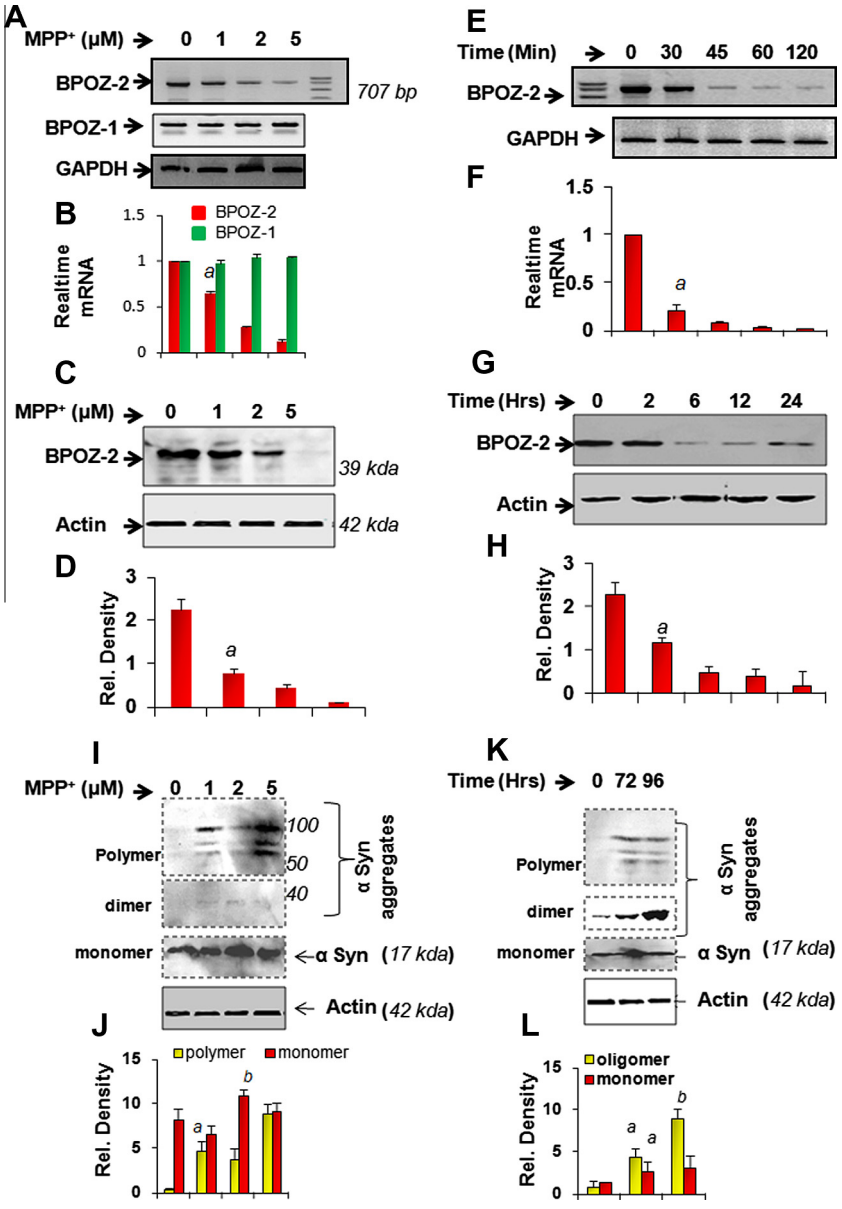

Fig. 1. Temporal relationship between BPOZ-2 and α-synuclein aggregation in MPP^+-stimulated mouse primary dopaminergic neurons. (A) In a dose-dependent study, mouse E14 DA neurons were treated with increasing doses of MPP^+ for 1 h and then tested for mRNA expression analyses of BPOZ-2 and BPOZ-1. Our mRNA analyses were further quantified with the realtime quantitative mRNA expression assay (B). $^aP < 0.05$ *vs.* control mRNA expression of BPOZ-2. (C) Similar to mRNA analyses, BPOZ-2 protein expression was quantified with increasing doses of MPP^+ after 12 h of stimulation with MPP^+ and that was further evaluated with a relative density plot (D). $^aP < 0.005$ *vs.* control BPOZ-2 protein (E) Next, mouse primary neurons were analyzed for BPOZ-2 mRNA expression at 0, 30, 45, 60, and 120 min after stimulation with 2 μM MPP^+. F is the realtime quantitative mRNA expression analyses of this time dependent experiment. $^aP < 0.01$ *vs.* control BPOZ-2 mRNA. In all cases, mRNA expression analyses were normalized with the GAPDH expression from respective samples. (G) DA neurons were immunoblotted for BPOZ-2 protein expression after 0, 2,6,12, and 24 h of stimulation with 5 μM MPP^+. (H) The plot represents the relative density of BPOZ-2 expression as shown in (G), measured as a ratio of BPOZ-2 and the respective beta-actin band density. $^aP < 0.01$ *vs.* control BPOZ-2 mRNA. (I) The protein expression of α-synuclein was analyzed in DA neurons at 1, 2, and 5 μM of MPP^+ stimulation for 72 h and further evaluated with the relative density analysis (J). Yellow bars represent the band densities of 100 Kda bands normalized with actin and red bars represent the monomeric expression of α-synuclein proteins normalized with respective β-actin bands. $^aP < 0.005$ *vs.* α-syn polymers in control cells and $^bP < 0.05$ *vs.* α-syn monomer in control cells. (K) MPP^+ simulates the expression of α-synuclein protein with its increasing time points that was further confirmed by densitometric analyses (L). Yellow bars show relative densities of 40 Kda α-syn dimeric bands whereas red bars represent monomeric bands. $^aP < 0.01$ *vs.* α-syn in control cells and $^bP < 0.005$ *vs.* α-syn oligomer in control cells results are ±S.D. of three independent experiments. GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase.

(Fig. 1E and F) with a dose of 5 μM of MPP^+ (Fig. 1A and B). According to our time dependent study, the mRNA expression of BPOZ-2 was completely abrogated at 2 h of stimulation with MPP^+ (Fig. 1B). Similarly, our immunoblot analyses revealed that MPP^+ inhibited the protein expression of BPOZ-2 with its increasing concentration (Fig. 1C) and time (Fig. 1G) with the lowest expression was achieved at 5 μM concentration and after 12 h of stimulation. The effect was further quantified by densitometric analyses as described under method section (Fig. 1D and H). Interestingly, in a similar dose (Fig. 1I and J) and time gradient (Fig. 1K and L) studies, we have detected the significant level of polymerized form of α-synuclein protein after 72 h of incubation of 5 μM MPP^+ as evident from our immunoblot analyses. The appearance of multiple high molecular weight bands between 60 and 100 Kda indicates the presence of polymerized α-synuclein molecules. However, the expression of 17 Kda monomeric α-synuclein protein was marginally upregulated in all different conditions as shown in our densitometric analyses (Fig. 1J and L) indicating that MPP^+ is primarily involved in the post-translation aggregation of α-synuclein molecules with trivial stimulation of transcription of *snca*gene. The dose responsive analysis revealed that the aggregation of α-syn (Fig. 1I and J) protein started at l μM concentration and reached maximum at 5 μM concentration. On the other hand, our time dependent analysis demonstrated that the aggregation started at 72 h of incubation peaking maximum at 96 h of MPP^+ incubation (Fig. 1K and L). Similarly, our immunofluorescence analysis revealed that the expression of BPOZ-2 was strongly downregulated at 6 h of stimulation reaching minimum at 12 h of stimulation with MPP^+ (Fig. 2A). Next we investigated the appearance of α-synuclein accumulation in DA neurons in the presence of MPP^+. The aggregated form of α-synuclein protein was observed to be appeared as a punctuated green signal at 72 h with maximum at 96 h of stimulation with MPP^+ (Fig. 2B). The effect was further supported with mean fluorescence intensity analyses as we observed that the load of BPOZ-2 signal was continued to decrease (Fig. 2C) with the late increase of α-synuclein burden in MPP^+-treated DA neurons (Fig. 2D), suggesting that the downregulation of BPOZ-2 could be involved for the accumulation of α-synuclein. Accordingly, our quantitative analyses further revealed that the number of DA neurons expressing BPOZ was significantly low after 6 h (Fig. 2E) whereas polymerized α-syn-immunoreactive cells were observed to be higher after 72 h and

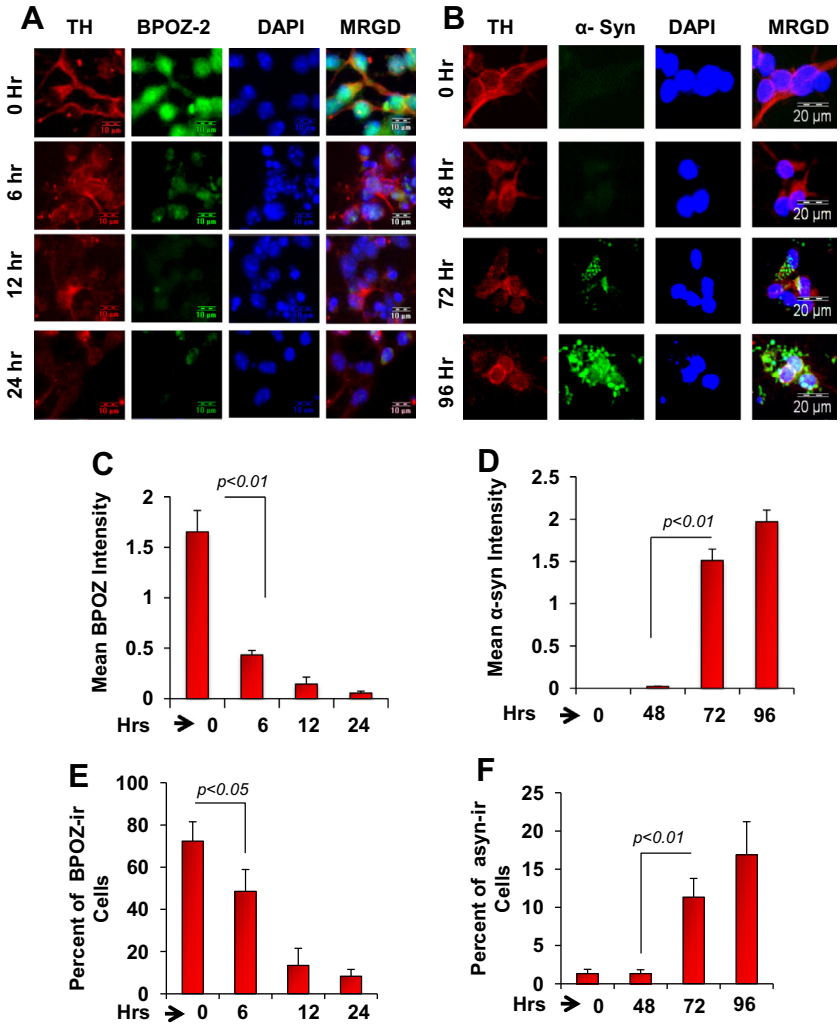

Fig. 2. Immunocytochemical analyses of BPOZ-2 and aggregated α-synuclein in MPP^+-stimulated mouse primary dopaminergic neurons. (A) Mouse primary neurons stimulated with 5 μM of MPP^+ for 0, 6, 12, and 24 h and then analyzed by immunocytochemical analyses of BPOZ-2 (green) and TH (red). (B) Mouse primary neurons were stained with α-synuclein (green) and TH (red) after 0, 48, 72, and 96 h of MPP^+ stimulation. Nuclei were stained with DAPI. Mean fluorescent intensities of BPOZ-2 (C) and α-synuclein (D) were analyzed by the microsuite 5 software of Olympus microscope. (E) Histogram represents the percent value of total number of BPOZ-2 expressing cells with respect to total number of DAPI-stained cells. (F) Histogram analyses of the percent of α-synuclein-immunoreactive cells versus total number of DAPI-positive cells with MPP^+ in different time points.

reached maximum at 96 h of MPP^+ stimulation (Fig. 2F). Taken together, our results suggest that the downregulation of BPOZ-2 expression might be involved in the aggregation of α-synuclein protein.

2.2. The necessary and sufficient role of BPOZ-2 in the amelioration of α-synucleinopathy

Next, we wanted to study the direct role of BPOZ-2 in the expression of α-synuclein in mouse primary DA neurons. To confirm the role of BPOZ-2, we performed siRNA mediated knocking down of BPOZ-2 (Fig. 3A) followed by the immunoblot analyses of α-synuclein in MPP^+-stimulated mouse primary DA neurons. Interestingly, our immunoblot (Fig. 3B) and immunofluorescence data (Fig. 3C) revealed that the selective knocking down of BPOZ-2 alone strongly upregulated the accumulation of α-synuclein oligomers equivalent to DA neurons treated with MPP^+ only. The effect was observed even higher when DA neurons were stimulated with BPOZ-2 siRNA along with MPP^+. Interestingly, the effect of BPOZ-2 siRNA on α-synuclein aggregation in DA neurons was observed to be marginally higher in the presence of MPP^+ (Fig. 3C and D), indicating that the specific ablation of BPOZ-2 is sufficient to establish α-synucleinopathy in DA neurons. This effect is stunning and to confirm that effect, we performed a dose dependent study in which mouse primary DA neurons were incubated with increasing concentrations of both control and *BPOZ-2*siRNA. Interestingly, we found that the increasing doses of BPOZ-2 siRNA, but not control siRNA, stimulated the appearance of punctuated α-synuclein inclusions at 72 h of incubation suggesting the direct role of BPOZ-2 in the abrogation of α-synuclein aggregates (Fig. 4A). Moreover, the quantitative analyses of our immunofluorescence results further revealed that BPOZ-2 siRNA, but not control siRNA, alone strongly stimulated the load of α-synuclein inclusions as well as the number of the α-synuclein-immunoreactive neurons in primary DA

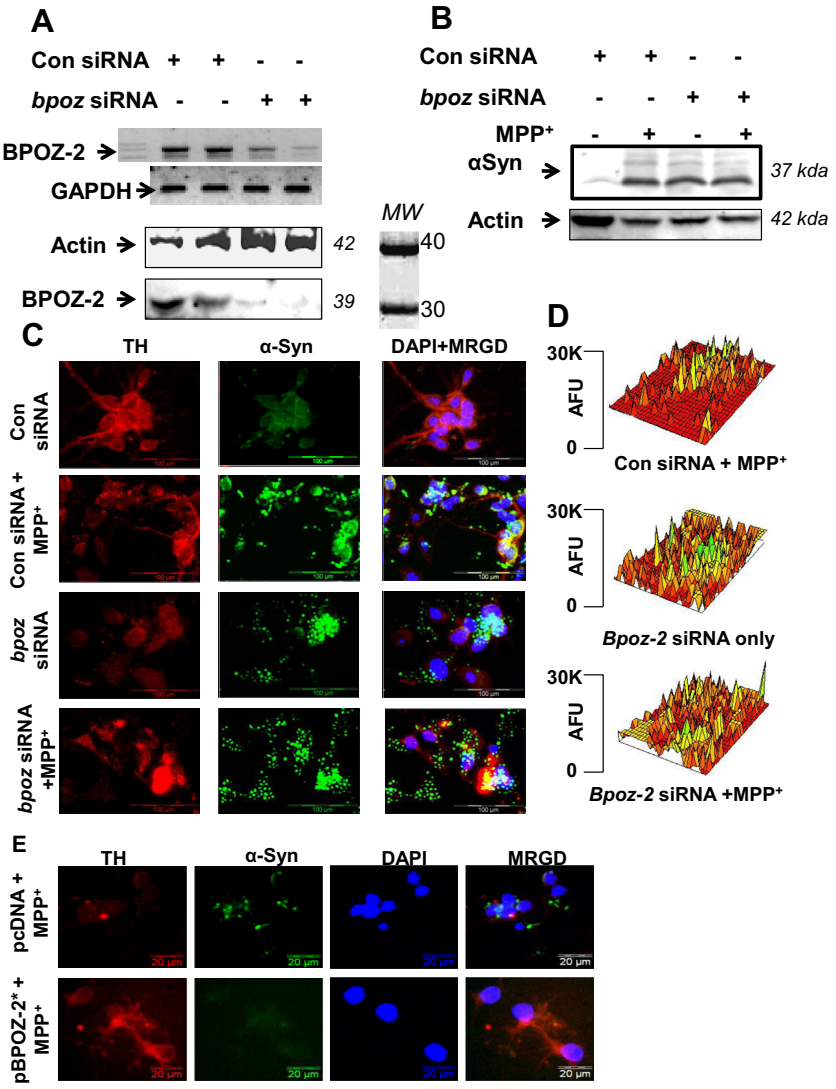

Fig. 3. Effect of BPOZ-2 siRNA on the accumulation of α-synuclein protein in MPP^+-stimulated mouse primary dopaminergic neurons. (A) Mouse primary DA neurons were stimulated with 0.5 μg of control and BPOZ-2 siRNA for 24 h followed by RTPCR *(top)* and immunoblot analyses *(bottom)* of BPOZ-2 expression to validate the efficiency of siRNA. Representative molecular weight marker shown in the panel was a size-matched Novex® Sharp Pre-Stained Protein Standard marker. (B) Immunoblot analyses of dimeric α-synuclein protein after 48 h of siRNA stimulation. Briefly cells were treated with siRNA for 24 h followed by the stimulation with MPP^+ for another 24 h. After that cells were blotted to check the oligomerization of α-synuclein protein. (C) Dual immunofluorescence analyses of TH (red) and α-syn (green) in DA neurons treated with control siRNA, BPOZ-2 siRNA, and siRNA plus MPP^+. (D) Three dimensional presentations of intensities of accumulated α-synuclein signal in a cell was plotted in arbitrary fluorescence unit (AFU) with the help of Image Dig software. Results are mean ±S.D. of three independent experiments. (E) Primary DA neurons were transfected with pcDNA and BPOZ-2 overexpression construct (Thermo fisher scientific, Cat #MMM 1013-202703602) for 24 h followed by the treatment of 2 μM of MPP^+ for another 24 h. After that cells were immunostained with TH (red) and α-syn (green). Nuclei are stained with DAPI.

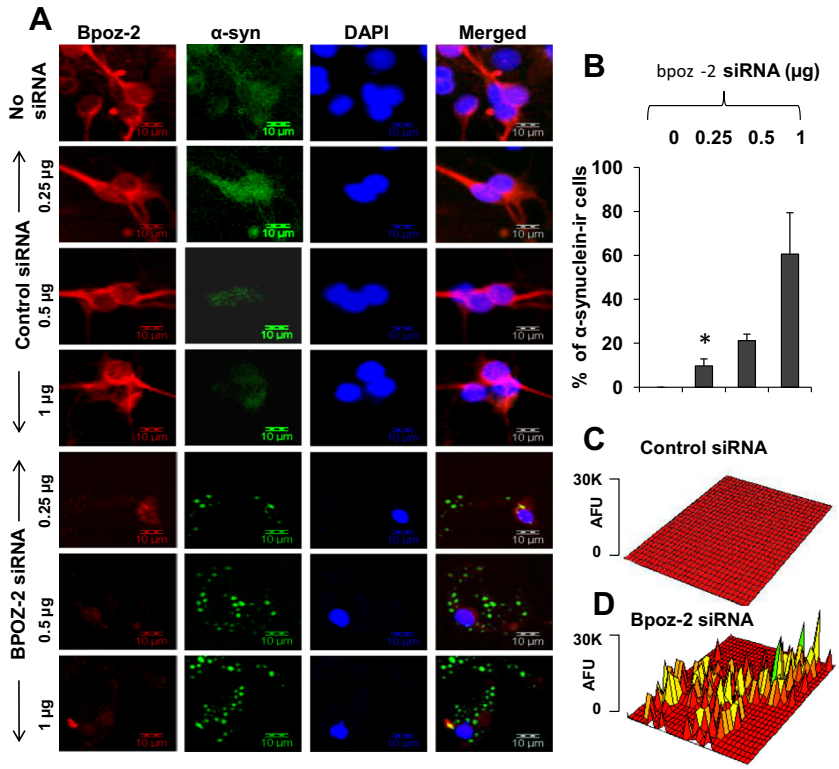

Fig. 4. Effect of BPOZ-2 siRNA alone on the upregulation of α-synuclein protein in mouse primary dopaminergic neurons. (A) Mouse primary DA neurons were stimulated with 0.25, 0.5, and 1 μg of control (top) and BPOZ-2 (bottom) siRNA for 48 h followed by immunofluorescence analyses of BPOZ-2 (red) and α-synuclein (green). Nuclei are stained with DAPI. (B) Percent of α-synuclein immunoreactive cells of total DAPI-positive cells was shown in the histogram. $^{*}P < 0.05$ *vs.* no siRNA treated cells. Counting was done in 25 independent images from each treatment group. (C) Fluorescence intensities of α-synuclein puncta in control siRNA and BPOZ-2 siRNA-treated cells were presented in 3D-plots.

neuronal culture (Fig. 4B and C). Taken together our results suggest that selective knockdown of BPOZ-2 is sufficient to generate α-synuclein aggregation in DA neurons. Next we wanted to study the effect of BPOZ-2 overexpression in the amelioration of α-synuclein aggregates in MPP^{+}-treated DA neurons (Fig. 3E). Interestingly, we observed that overexpression of BPOZ-2 significantly attenuated the accumulation of α-synuclein in MPP^{+}-treated DA neurons further suggesting the essential and pivotal role of BPOZ-2 in the prevention of α-synucleinopathy in DA neurons.

2.3. BPOZ-2 inhibited the aggregation of α synuclein via post-transcriptional, but not transcriptional modification

So far, we observed that the selective knockdown of *BPOZ-2* primarily elevated the aggregation of α-synuclein protein in mouse primary DA neurons. Since accumulation of α-synuclein is also caused by the increased transcription of snca gene, next we wanted to study if BPOZ-2 at all plays any role in the transcriptional inhibition of α-synuclein gene in order to reduce the burden of α-synuclein aggregates in DA neurons. In order to test that possibility, we first performed both semiquantitative and quantitative mRNA analyses of α-synuclein in control siRNA- and BPOZ-2 siRNA-treated neurons (Fig. 5A) at 24, 48, and 72 h of stimulation with MPP^{+}. Interestingly we observed that BPOZ-2 siRNA was unable to induce the level of α-synuclein mRNA in DA neurons as evident from our semiquantitative RT-PCR analyses (Fig. 5 A). The effect was further confirmed by quantitative realtime PCR analyses (Fig. 5B and C). Taken together our results suggest that BPOZ-2 is only involved in the post-transcriptional stability, but not in the transcription of α-synuclein molecule and thereby leads to the late formation of α-synuclein aggregates in neuron.

2.4. BPOZ-2 inhibited the aggregation of α synuclein in vivo in nigra of MPTP-intoxicated vertebrate model of Parkinson's disease

So far, we analyzed the effect of BPOZ-2 on the formation of α-synuclein aggregates in mouse primary DA neuronal cultures and that effect could be different *in vivo* in the brain. Therefore, next we wanted to study the effect of BPOZ-2 on the accumulation of α-syn *in vivo* in the nigra of MPTP-intoxicated mouse brain. First, we performed a double immunofluorescence analysis of α-synuclein (green) and BPOZ-2 (red) in the nigra of mouse brain. We observed strong downregulation of BPOZ-2 expression in the DA neurons in the nigra of MPTP-treated mice, which was coupled with the elevated level of α-synuclein aggregates suggesting the negative correlation between BPOZ-2 expression and α-synucleinopathy *in vivo* in the nigra of mouse brain (Fig. 6A). According to our immunofluorescence analyses, we observed that the number of BPOZ-2 positive and α-synuclein negative cells are higher in the control brain and are significantly low in MPTP-treated mouse nigra as shown in red colored cells. On the other hand, we observed significantly higher number of α-synuclein positive but BPOZ-2 negative cells in the nigra of MPTP-treated mouse brain than the nigra of control brain as shown in green colored cells suggesting the essential role of BPOZ-2 in reducing the burden of α-synuclein *in vivo* in brain. Since the load of α-synuclein aggregates has been reported to be high in the brain of chronic animal model of PD, next we performed the double immunofluorescence analysis of BPOZ and α-synuclein in the nigra of MPTP-induced chronic mouse model of PD. As expected, we observed that nigra of chronic brain expressed relatively higher loads of α-synuclein than acute nigra with fewer cells expressing BPOZ-2. Next, we wanted to validate our finding in MPTP-induced hemiparkinsonian primate model of

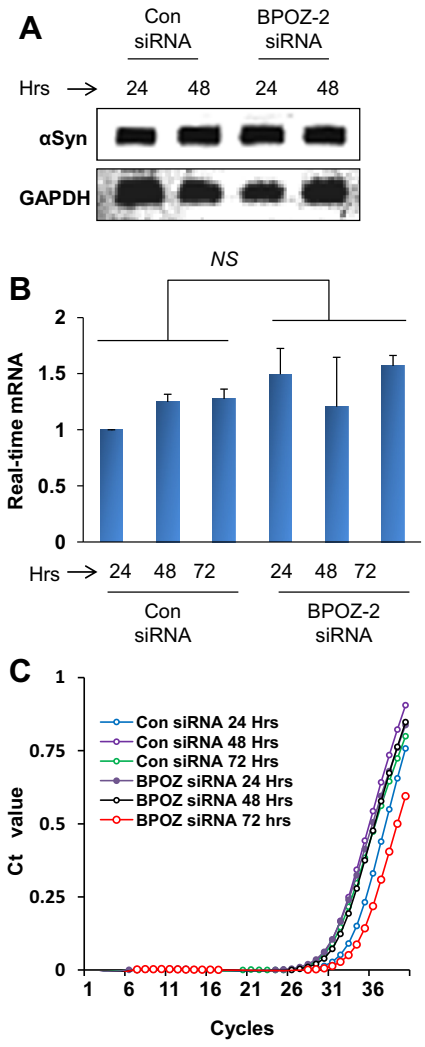

Fig. 5. SiRNA knockdown of BPOZ-2 was unable to stimulate the mRNA expression of snca gene in in mouse primary dopaminergic neurons. (A) Mouse primary DA neurons were stimulated with 0.5 μg of control and BPOZ-2 siRNA for 24, 48 and 72 h followed by the semiquantitative RT-PCR (A) and quantitative realtime PCR (B) analyses of *snca* gene. (C) Ct Value of the respective realtime data was plotted to demonstrate that there was no difference in the expression of *snca* gene.

PD. Similar to our mouse model, we observed that TH neurons expressed significant amount of BPOZ-2 and the level decreased when animals treated with MPTP. Moreover, we observed that neurons containing the α-synuclein aggregate were totally devoid of BPOZ-2 as shown by arrow head. Taken together, our results indicate that BPOZ-2 indeed inhibits the aggregation of α-synuclein *in vivo* in the nigra of MPTP-induced mouse brain of PD.

3. Discussion

Cytoplasmic aggregation of α-synuclein protein is the pathological hallmark PD as it inhibits the intracellular transport of different proteins and metabolites leading to the axonal degeneration and death of nigrostriatal DA neurons [15,16]. Therefore, the removal of α-synuclein aggregates is a potential therapeutic challenge in the treatment of PD. So far, the dysfunction of proteasome–ubiquitin machinery in DA neurons is believed to be a major contributing factor for the α-synucleinopathy and hence, the ubiquitin enzyme complex is always under scanner in search of the molecular mechanism for α-synuclein aggregation. However, there is not much known about the ubiquitination machinery involved in the degradation of α-synuclein protein. Our study first time reveals the involvement of BPOZ, an E3 ubiquitin ligase associated protein, in the early events of α-synuclein aggregation. However, it would be premature to comment that BPOZ-2 is involved in the degradation of α-synuclein via ubiquitin–proteasomal pathway, which requires much rigorous experimental evidences. The role of BPOZ protein in the pathogenesis of cancer has been reported before. However, its role in the pathogenesis of neurodegenerative disorders has not been studied so far. Our present manuscript first time reports the detection of this molecule in the DA neurons and also highlights its protective role in the loss of DA neurons in PD. Our results clearly demonstrate that BPOZ plays a critical role in the survival of DA neurons in presence of PD pathology as its expression is strongly downregulated in presence of MPP^+. The effect precedes much ahead of the accumulation of α-synuclein aggregates in DA neurons indicating that its inhibition might be responsible for the accumulation of α-synuclein protein in DA neurons. Our time-dependent analyses of bpoz mRNA and protein expression clearly indicate that the downregulation of BPOZ has a strong correlation with the accumulation of α-synuclein protein. In agreement with this fact, BPOZ siRNA alone stimulated the building up of α-synuclein oligomers in DA neurons suggesting the direct involvement of BPOZ in the α-synucleinopathy. While looking at the mechanism, we observed that the siRNA knockdown of BPOZ did not alter the mRNA expression of *snca* gene indicating that BPOZ is not involved in the transcriptional regulation. Instead, BPOZ is involved in the post translation regulation of α-synuclein protein as our immunoblot analyses under similar treatment condition significantly increased the cellular burden of α-synuclein protein in the cultured DA neurons. Since, BPOZ is a well-known co-activator of E3 ubiquitin ligase that controls the turnover of many biological proteins; it is obvious that similar mechanism of protein degradation by BPOZ is impaired in MPP^+-treated DA neurons that leads to the formation of α-syn aggregates. Similar to our cell culture studies, we observed that BPOZ expression was strongly downregulated in the nigra of both acute and chronic mouse model as well as in the primate model of PD. In all cases, strong downregulation of BPOZ is accompanied with the elevated expression of α-synuclein aggregates validating our hypothesis *in vivo* in animal model.

BPOZ is an ankyrin rich scaffold protein that has been known to anchor E3 ubiquitin ligases in order to catalyze the degradation of the tumorigenic protein. Until now, no study had been done to study its role in the regulation of neuronal function. It is not known if this protein is involved in the survival and growth of neurons in healthy as well as diseased brain. In that context, our present study introduces BPOZ as a neuroprotective protein of DA neurons when they are challenged to death in PD. Another strength of our paper is that we have validated our finding in vivo in different rodent and primate model of PD. We have successfully shown that the downregulation of BPOZ-2 is truly involved in the nigral accumulation of α-synuclein. The degradation mechanism and proteins associated for the degradation of α-synuclein is poorly understood. In that sense our study highlights the possible role of BPOZ-2 protein in the degradation of α-synuclein. However, we do not know if this molecule modulates the action of parkin, a PD associated E3 ubiquitin ligase. However, recent reports suggest that parkin interacts with CUL family of E3 ubiquitin ligase [17], which is a well-known binding partner of BPOZ-2 [2,5]. It can be predicted that there might be a crosstalk between BPOZ-2 and parkin via CUL family of ubiquitin ligase. Therefore, the association of parkin with BPOZ is definitely an area of our future interest and if we observe the association of BPOZ and parkin modulates the catalytic action of parkin, then that will certainly explain the reason for the dysfunction of parkin protein in presence of PD pathology.

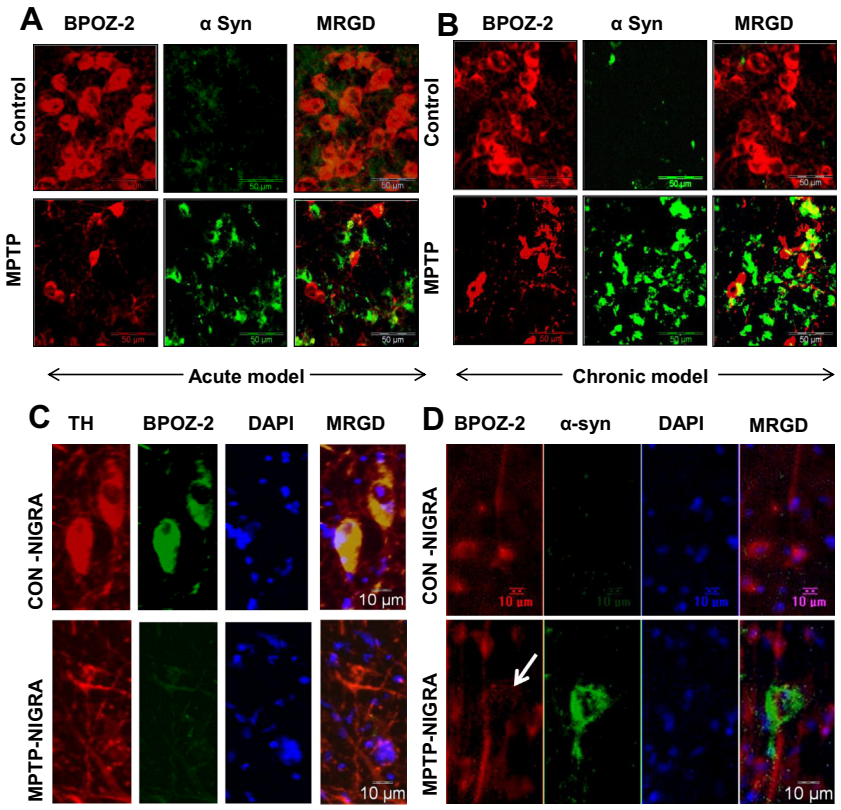

Fig. 6. The role of BPOZ-2 in the accumulation of α-synuclein protein in the nigra of MPTP-induced mouse and primate model of PD. Dual immunofluorescence analyses of BPOZ-2 (red) and α-syn (green) in the nigra of control and MPTP-intoxicated mice brains in acute (A) and chronic model (B) of PD. (C) Expression of BPOZ-2 (green) was analyzed in TH-immunoreactive (red) nigra of MPTP-induced hemiparkinsonian monkey brain. (D) Dual immunofluorescence analyses of BPOZ-2 (red) and α-syn (green) in the nigra of control and hemiparkinsonian monkey brain. Arrow sign indicates the neurons deficient with BPOZ-2 is associated with the accumulation of α-synuclin protein.

4. Materials and methods

4.1. Animals and reagents

Male C57 BL/6 mice were purchased from Jackson Laboratory and maintained in the animal facility of Rush University Medical Center. BPOZ-2 siRNA (Cat #sc-140786) was obtained from Santacruz biotechnology, BPOZ-2 antibody (Cat #ab1077) was purchased from Abcam, primers of RT-PCR and lipofectamine200 transfection reagents were purchased from Invitrogen.

4.2. Isolation of primary dopaminergic neurons

Nigra was dissected as a thin slice of ventral mesencephalon tissue from E12.5 to 14 days old fetus and will be homogenized with 1 ml of trypsin for 5 min at 37 °C followed by neutralization of trypsin [18]. The single cell suspension of nigral tissue was plated in the poly-D-lysine pre-coated 75 mm flask and was allowed to differentiate fully for 9–10 days before treatment [18–20].

4.3. Semiquantitative RT-PCR

Total RNA was isolated from DA neurons using Ultraspec-II RNA reagent (Biotecx Laboratories, Inc., Houston, TX) following manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase. RT-PCR was carried out as described earlier [21,22] using a RT-PCR kit (Clontech, Mountain View, CA) with following primers. BPOZ-2 sense 5′GTGGGCAT GGAACAGTGAGA3′ and antisense 5′TGTCCACCATCCAACGGAAG3′. Seven hundred seven bp long product was electrophoresed in ethedium bromide containing 1.5% agarose gel and detected in our bio-rad gel doc machine.

4.4. Quantitative realtime PCR

Quantitative RT-PCR was performed using the ABI-Prism7700 sequence detection system (Applied Biosystems) as described earlier [23]. Briefly, reactions were performed in 96-well optical reaction plates on cDNA equivalent to 50 ng of DNase-digested RNA in a volume of 25 μl, containing 12.5 μl of SYBR Green Universal Master mix, nuclease free H_2O, and forward and reverse primers following manufacturer's protocol. Primers of BPOZ-1, BPOZ-2, and GAPDH were purchased from Invitrogen. The mRNA expression of BPOZ-2 was normalized to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA. Data were processed by the ABI Sequence Detection System 1.6 software and analyzed by analysis of variance.

4.5. SiRNA knockdpon of BPOZ-2

Mouse primary DA neuronal culture was transfected with 0.25–0.5 μg of BPOZ-2 siRNA using lipofecatmine 2000 following manufacturers' protocol under serum free condition. The efficiency of transfection was increased with the use of neupherin reagent. After 4 h of transfection neurons were supplemented with serum enriched media and after another 24 h neurons were treated with MPP^+.

4.6. Immunofluorescence analysis

Immunocytochemistry was performed as described earlier [24,25]. Briefly, coverslips containing neurons cultured to 70–80% confluence were fixed with chilled methanol (Fisher Scientific, Waltham, MA) overnight, followed by two brief rinses with filtered PBS. Samples were blocked with 2% BSA (Fisher Scientific) in PBS

containing Tween 20 (Sigma–Aldrich) and Triton X-100 (Sigma–Aldrich) for 30 min and incubated at room temperature under shaking conditions for 6 h in PBS containing the following anti-mouse primary Abs: BPOZ-2 (1:200; Cat #ab1077 Abcam), α-synuclein (1:2000; Cat #610787 BD transduction laboratories), TH (1:1000, Calbiochem). After four 15-min washes in filtered PBS, slides were further incubated with Cy2- or Cy5-labeled secondary Abs (all 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h under similar shaking conditions. Following four 15-min washes with filtered PBS, cells were incubated for 4–5 min with DAPI (1:10000; Sigma–Aldrich). The samples were run in Xylene (Fisher Scientific) gradient, mounted, and observed under a Bio-Rad MRC1024ES confocal laser-scanning microscope.

4.7. Immunoblot analysis

Immunoblot analysis was conducted as described earlier [25]. Briefly, cells were scraped in lysis buffer, analyzed for protein concentration via the Bradford method (Bio-Rad). SDS sample buffer was added to 40–60 μg total protein and boiled for 5 min. Denatured samples were electrophoresed on NuPAGE Novex 4–12% Bis–Tris gels (Invitrogen), transferred onto a nitrocellulose membrane (Bio-Rad) using the Thermo-Pierce Fast Semi-Dry Blotter, washed for 15 min in TBST, blocked for 1 h in TBST containing BSA. Next, membranes were incubated overnight at 4 °C under shaking conditions with primary Abs followed by thorough wash and incubation with 2° Abs for 1 h at room temperature, washed for one more hour, and visualized under the Odyssey Infrared Imaging System (Li-COR, Lincoln, NE). Relative density of immunoblots was measured by taking individual pixel value followed by the division with their respective actin value in image J software suite.

4.8. Development of different animal models used for this study

Development of chronic mouse model was described earlier[26]. In this model, six- to eight-week-old male C57BL/6 mice received 10 injections of MPTP (subcutaneously [s.c.]; 25 mg/kg body weight) together with 10 injections of probenecid (intraperitoneally [i.p.]; 25 mg/kg body weight) for five weeks. For acute model, mice received four intraperitoneal injections of MPTP-HCl (18 mg/kg of free base; Sigma–Aldrich) in saline at 2 h intervals [27,28]. The development of hemiparkinsonian monkey model was described elsewhere[29]. Animal maintaining and experiments were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Rush University Medical Center.

4.9. Statistical analysis

Values are expressed as means ± S.D. of at least three independent experiments. Statistical analyses for differences were performed via one-way ANOVA, followed by Tukey's or Scheffe's post hoc tests using SPSS 19 (IBM, Armonk, NY). The criterion for statistical significance was $P < 0.05$.

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